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INCORPORATION OF IN VITRO METABOLISM DATA IN A PHYSIOLOGICALLY BASED PHARMACOKINETIC (PBPK) MODEL FOR CHLOROPRENE

ABSTRACT

A physiologically based pharmacokinetic (PBPK) model for chloroprene in the mouse, rat and human has been developed that relies solely on in vitro studies for the estimation of model parameters describing tissue metabolism and partitioning. The PBPK model accurately predicts in vivo pharmacokinetic data from a 6-hr, nose-only chloroprene inhalation study conducted with female B6C3F1 mice, the most sensitive species/gender for lung tumors in the 2-year bioassays conducted with chloroprene. This PBPK model has been developed to support an inhalation cancer risk assessment for chloroprene using in vitro data on the metabolism of chloroprene to reactive epoxides in the lung target tissue of mice and humans. The approach for calculating target tissue (lung) dose metrics was based on the PBPK modeling performed in support of the inhalation cancer risk assessment for methylene chloride and represents the best available science for determining the impact of species differences in metabolism of chloroprene.

Keywords: chloroprene, inhalation, PBPK, cancer risk assessment

1. INTRODUCTION

Chloroprene (CAS # 126-99-8) is a highly volatile chlorinated analog of 1,3-butadiene that is used in the manufacture of polychloroprene rubber (Neoprene). A cancer risk assessment for chloroprene conducted by the USEPA (2010) calculated an inhalation unit risk (IUR) of 5×10^{-4} per $\mu\text{g}/\text{m}^3$ based on tumor incidence data from female mice exposed to chloroprene for 2 years (NTP 1998; Melnick et al. 1999). The USEPA (2010) assessment used a default cross-species extrapolation approach based on chloroprene exposure concentration, despite strong evidence of quantitative differences in chloroprene metabolism in mice and humans that would have a significant impact on the calculated risk (Himmelstein et al. 2004a, 2004b). The metabolism of chloroprene results in the formation of reactive epoxides that are considered to be responsible for its carcinogenicity in rodents (USEPA 2010).

To determine the potential impact of species-specific differences in the production of these epoxides, a physiologically based pharmacokinetic (PBPK) model was developed in a collaborative research effort between DuPont Haskell Laboratory and the USEPA National Health and Environmental Effects Research Laboratory (NHEERL). In vitro measurements of partition coefficients and metabolism parameters for chloroprene in mice, rats, hamsters and humans (Himmelstein et al. 2004a) were used in the PBPK model (Himmelstein et al. 2004b) to predict species-specific dose metrics for the production of epoxides in the lung, the most sensitive tissue in the mouse bioassay. The dose metric chosen for this comparison is consistent with the dose metrics used in previous PBPK-based risk assessments for methylene chloride and vinyl chloride, which are also metabolized to reactive metabolites that are considered to be responsible for the observed carcinogenicity in rodents. Closed-chamber exposures of mice, rats and hamsters were used to validate the PBPK model's ability to predict the pharmacokinetic behavior of chloroprene in vivo. The USEPA (2010), however, did not make use of the PBPK model from Himmelstein et al. (2004b) in their risk assessment, citing the lack of blood or tissue time course concentration data for model validation. In addition, USEPA indicated that they did not consider the comparisons of model predictions with the closed-chamber studies to be adequate because the data were limited to chloroprene vapor uptake from the closed chambers.

After the time of the USEPA (2010) evaluation, Yang et al. (2012) provided additional data for refining the PBPK model of Himmelstein et al. (2004b). To supplement the data in Himmelstein et al. (2004a) on liver and lung metabolism in male mouse, male rat, and pooled human cells, Yang et al. (2012) measured liver and lung metabolism in female mouse and female rat, as well as kidney metabolism in male and female mouse, male and female rat, and pooled human cells. The totality of the data from the Himmelstein et al. (2004a) and Yang et al. (2012) in vitro metabolism studies was then used to refine the metabolism parameter estimates for the chloroprene PBPK model using Markov-chain Monte Carlo (MCMC) analysis. A comparison of lung dose metric estimates in mouse, rat and human was then performed using the updated metabolism parameters (Yang et al. 2012). These dose metrics were subsequently used in a study comparing genomic responses to chloroprene in the mouse and rat lung (Thomas et al. 2013) and a study comparing human risk estimates derived from mouse bioassay and human epidemiological data (Allen et al. 2014), but to date no in vivo blood or tissue time course concentration data have been published with which to evaluate the ability of the chloroprene PBPK model to predict in vivo kinetics.

The objectives of the present study were to: 1) characterize the in vivo pharmacokinetics of chloroprene via analysis of arterial whole blood concentrations in female B6C3F1 mice during and

following a single 6-hour nose-only inhalation exposure, and 2) determine respiratory parameters (breathing frequency and tidal volume) during chloroprene exposure. In this paper we also demonstrate the ability of the refined chloroprene PBPK model to reproduce new in vivo validation data and calculate PBPK dose metrics to support an inhalation cancer risk assessment that properly considers species differences in pharmacokinetics and metabolism.

2. MATERIALS AND METHODS

Nose-only Exposure Study

Test Substance and Atmosphere Generation

The test substance, β -Chloroprene (CAS # 126-99-8) containing polymerization inhibitors, was supplied by the sponsor as a clear liquid. Exposure atmospheres were generated by metering saturated chloroprene vapor from a stainless-steel pressure vessel reservoir (McMaster Carr, Atlanta, GA) into the nose-only exposure chamber air supply. The concentrated chloroprene vapor was metered through a mass flow controller (MKS Instruments Inc., Andover, MA) and mixed with HEPA-filtered air approximately six feet upstream of the nose-only inlet. Chloroprene vapor was introduced counter-current to the dilution air to facilitate mixing of the vapors with the dilution air. Chloroprene concentrations were monitored on-line using a gas chromatography system with flame ionization detector (GC-FID). Calibration of the GC-FID for chloroprene analysis was conducted through the analysis of a series of calibration standards produced by introducing pure chloroprene into Tedlar® bags containing known volumes of nitrogen gas (nitrogen was metered into the bag using a calibrated flow meter).

Test Animals and Housing

Female B6C3F1 were purchased from Charles Rivers Laboratories, Inc (Raleigh, NC) at 8 weeks of age and acclimated to their surroundings for approximately two weeks prior to use. Following acclimation animals were assigned to a dosing group by randomization of body weights using Provantis NT 2000, assigned unique identification numbers, cage cards, and housed (1/cage) in polycarbonate cages with standard cellulose bedding. Animals were housed in a humidity and temperature controlled, HEPA-filtered, mass air-displacement room provided by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited animal facility at The Hamner Institutes. This room was maintained on a 12-hour light-dark cycle at approximately 64oC-79oF with a relative humidity of approximately 30-70%. Rodent diet NIH-07 (Zeigler Brothers, Gardners, PA) and reverse osmosis water was provided ad libitum except during exposures. Food and water were withheld from all animals during the chloroprene exposures. Prior to the start of the chloroprene exposure, animals were weighed, and their weights were recorded.

The Hamner Institutes for Health Sciences was fully accredited by the AAALAC during the time the study was performed. Currently acceptable practices of good animal husbandry were followed per the National Research Council Guide for the Care and Use of Laboratory Animals and were in compliance with all appropriate parts of the Animal Welfare Act. In addition, the study design and protocol were approved by The Hamner Institutes' Institutional Animal Care and Use Committee (IACUC) prior to the initiation of the study.

Inhalation Exposures

Inhalation exposures were conducted at 13, 32, and 90 ppm for 6 hours. Arterial blood was collected at a total of 6 time-points, 0.5, 3, and 6 hours during exposure and 5, 10, and 15 minutes post-exposure. To support collection of whole blood during the exposures, nose only towers were fitted with specially designed nose only exposure tubes. These exposure tubes were manufactured from 50 mL polypropylene bulb irrigation syringes (Sherwood Medical, St. Louis, MO). Three elongated holes (0.625" x 1.125") were drilled into the wall of the syringe to allow access to the thorax of the mouse during chloroprene exposure. A second irrigation syringe was

cut to form a sleeve around the first syringe to provide an air tight barrier during the exposures. This sleeve was pulled back during the exposure to allow for the injection of pentobarbital (100 mg/kg) while the animal continued to inhale chloroprene. Arterial blood was removed directly from the mouse via cardiac puncture while the mouse was still housed in the syringe and breathing chloroprene.

Plethysmography

A total of 16 mice (4 per exposure group including air controls) were used for the purpose of collecting tidal volume and breathing frequency. Data were acquired using modified nose-only Buxco plethysmograph tubes for pulmonary function monitoring. Data from control mice were collected prior to the first chloroprene exposure. Plethysmography data from both control and exposed mice were collected for 2-3 hours.

Blood Sampling

Collection of whole blood from chloroprene exposed mice was conducted using nose only towers and specially designed nose only exposure tubes. Whole blood was collected at 0.5, 3, and 6 hours during exposure and 5, 10, and 15 minutes post-exposure. Whole blood collection during chloroprene exposures (0.5, 3, and 6-hour time points) were done using the specially designed nose only exposure tubes mentioned in sections 3.2.1 and 3.2.2 above.

Blood Analysis

Quantification of chloroprene in whole blood was conducted by headspace sampling with analysis by gas chromatography mass spectrometry (GC/MS). The sampling method to be used, headspace analysis, as well as the GC/MS method were based on the previously published method for the analysis of 1,3-butadiene in whole blood from mice and rats (Himmelstein et al. 1994).

Briefly, 200 µL of whole blood, obtained by cardiac puncture, was transferred into pre-labeled, capped, and weighed airtight headspace vials (1.5 mL autosampler vial). Sample vials were weighed to obtain an accurate estimate of sample size and allowed to equilibrate at room temperature for 2 hours. Once equilibration was complete, samples were analyzed using an Agilent 5973 mass spectrometer coupled to an Agilent 6890 gas chromatograph. The mass spectrum was run in electron impact mode with selective ion monitoring (instrumental conditions are listed below).

Calibration curves were prepared by spiking stock control whole blood with known amounts of chloroprene obtained as a certified standard solution of chloroprene in methanol. Quality control samples were prepared by spiking control rat plasma with a certified chloroprene standard. QC samples were spiked to low (near the first calibration point), medium (near the middle of the calibration curve), and high (near the highest point of the calibration curve) levels. Aliquots of the prepared QC's were placed in sealed GC vials (3 aliquots for each level, 9 total) and kept frozen at -80°C until required (GC vials had a minimum of headspace prior to freezing). On the blood collection days, a low-, middle-, and high-level QC was thawed and allowed to come to room temperature for 4 hours. After this time, the QC samples were "sampled" with a syringe identical to those being used for the collection of whole blood, placed in a GC vial in a manner identical to that of the whole blood collection, and analyzed along with the samples and standards.

Additional details of the nose-only inhalation study can be found in IISRP (2009a).

Chloroprene PBPK Model

Model Structure

The structure of the PBPK model used in this study (Figure 1) is based on the PBPK model of chloroprene described in Himmelstein et al. (2004b), as modified by Yang et al. (2012). As in previous models of volatile organic compounds (Ramsey and Andersen 1984, Andersen et al. 1987), the blood is described using a steady-state approximation and the model assumes blood-flow limited transport to tissues and venous equilibration of tissues with the blood. Metabolism is described in the liver, lung and kidney using Michaelis-Menten saturable kinetics.

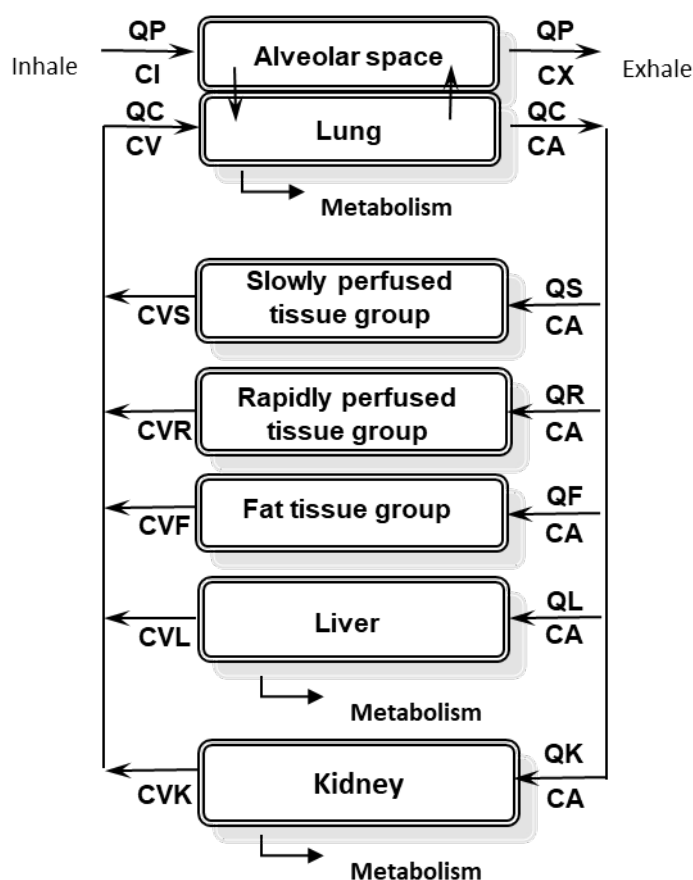


Figure 1. Chloroprene PBPK model diagram. Abbreviations: QP - alveolar ventilation; CI - inhaled concentration; CX - exhaled concentration; QC - cardiac output; CA - arterial blood concentration; CV - venous blood concentration; QS , CVS - blood flow to, and venous concentration leaving, the slowly perfused tissues (e.g. muscle); QR , CVR - blood flow to, and venous concentration leaving, the richly perfused tissues (most organs); QF , CVF - blood flow to, and venous concentration leaving, the fat; QL , CVL - blood flow to, and venous concentration leaving, the liver; QK , CVK - blood flow to, and venous concentration leaving, the kidney.

Model Parameters

All physiological parameters in the model for mouse, rat and human (Table S-1 in Supplemental Materials A) are taken from Brown et al. (1997) except for the cardiac output in the mouse and

the alveolar ventilation and cardiac output in the human. While the alveolar ventilation in the mouse is taken from Brown et al. (1997), relying on the value of cardiac output reported in Brown et al. (1997) would result in a value of 11.6 L/hr/bw^{3/4} for cardiac output (QCC). If used with the Brown et al. (1997) value of 29.1 L/hr/bw^{3/4} for alveolar ventilation (QPC), this would result in a serious mismatch between ventilation and perfusion (V/Q ratio $\gg 1$). Andersen et al. (1987), the developers of the PBPK model for methylene chloride that was used in the USEPA (2011) IRIS assessment, argued that it would be more biologically realistic to assume that the V/Q ratio was close to 1 at rest, and stated that their previous experience with PBPK modeling of data on clearance of chemicals in the mouse under flow-limited metabolism conditions supported the use of a higher value for QCC. Therefore, the value of QCC in the current model was calculated by dividing the alveolar ventilation from Brown et al. (1997) by MCMC estimates of a V/Q ratio for the mouse based on pharmacokinetic data for exposures to another volatile organic chemical, methylene chloride (Marino et al. 2006), which was used in the USEPA (2011) inhalation cancer risk assessment for that chemical. In the case of the human, it is more appropriate to use the default EPA ventilation rate of 20 L/day, reflecting an average activity level, rather than a resting value (Clewett et al. 2001). Since the values for alveolar ventilation and cardiac output in Brown et al. (1997) are resting values, we used the values calculated for the PBPK model of vinyl chloride (Clewett et al. 2001), which was developed for the USEPA (2000) cancer risk assessment for that chemical.

Apart from the physiological parameters, the model parameters are based entirely on in vitro data. The partition coefficients (Table S-2 in Supplemental Materials A) were calculated from the results of in vitro vial equilibration data reported by Himmelstein et al. (2004b), using the partition coefficients for muscle and kidney to represent the slowly and rapidly perfused tissues, respectively. To obtain the model parameters for metabolism in the liver, lung and kidney, the original in vitro chloroprene metabolism time-course data (Himmelstein et al. 2004a, IISRP 2009b) were re-analyzed using a MCMC analytical approach similar to the one performed in Yang et al. (2012). The key differences between the new analysis and the original Yang et al. (2012) analysis were: (1) the incorporation of an additional parameter in the analysis of the in vitro metabolism data (K_{gl}) to describe the rate of transfer of chloroprene from the headspace to the media in the metabolism studies, (2) the use of updated tissue microsomal protein concentrations for scaling the in vitro results to in vivo values appropriate for the PBPK model, and (3) the adoption of a previously published approach for estimating the metabolism parameters in the human lung (Andersen et al. 1987).

Re-estimation of in vitro metabolism parameters: Schlosser et al. (1993) suggested that mass transport limitations should be assessed when estimating metabolism from in vitro experiments conducted with volatile compounds where there is an air:liquid interface. Since mass transport limitation was not addressed in the in vitro metabolism studies conducted with chloroprene (Himmelstein et al. 2004a, Yang et al. 2012), a new experimental study was performed to estimate a K_{gl} for chloroprene following a protocol like that in Schlosser et al. (1993). The new experimental study, which is described in Supplemental Materials B, resulted in an estimated value of 0.024 L/hr for K_{gl}, similar to the value previously reported for benzene (Schlosser et al. 1993). However, the experimental value of K_{gl} was not consistent with the high rates of liver metabolism observed at low concentrations of chloroprene; that is, the mass transport associated with a K_{gl} of 0.024 L/hr was too slow to support the observed rates of metabolism in the media.

We considered it likely that the much faster uptake of chloroprene in the metabolism studies than in the K_{gl} study was due to more effective mixing during the incubations, together with non-

specific surface binding of chloroprene to the microsomes, which provide a lipophilic binding component in the aqueous media. No microsomes were present in the K_{gl} experiments for chloroprene or benzene (Schlosser et al. 1993). Although the rate of shaking in the metabolism studies was not reported, we were able to determine that the Himmelstein et al. (2004a) and IISRP (2009b) studies used a Gerstel MPS2 autosampler with an agitating heater, which was set to an agitation rate of 500 rpm (Matt Himmelstein, personal communication). Based on this information, it was suggested (Paul Schlosser, personal communication) that the value of K_{gl} in the metabolism studies was likely to be higher than the value in the new experimental study by roughly the ratio of the mixing rates, that is, $K_{gl}(\text{metabolism studies}) = K_{gl}(\text{experimental study}) \times 500/60 = 0.024 \times 500/60 = 0.2 \text{ L/hr}$. To confirm this expectation, we conducted a new MCMC analysis to simultaneously estimate K_{gl}, V_{max} and K_m from the metabolism data for the male mouse (Himmelstein et al. 2004a), which provided the strongest information regarding the dose-response for the clearance of chloroprene in the vials. The resulting value of K_{gl} estimated from this analysis was 0.22 L/hr, with a 95% confidence interval of 0.19 – 0.33 L/hr, consistent with the estimated value. The estimated value was then used in the re-estimation of the metabolism parameters for all tissues (Supplemental Materials B). The results of the new in vitro metabolism parameter estimation are provided in Table S-3 in Supplemental Materials A.

Selection of tissue scaling parameters: Based on a review of the literature (Supplemental Materials C), an updated set of scaling parameters was chosen: 35, 45, and 40 mg protein/g liver for mice, rats, and humans, respectively, 20 mg protein/g lung for all species, and 11 mg protein/g kidney for all species (Medinsky et al. 1994, Houston and Galetin 2008, Barter et al. 2008, Yoon et al. 2007, Scotcher et al. 2017). The in vivo metabolism parameters derived using the revised scaling parameters are listed in Table S-4 in Supplemental Materials A and the IVIVE calculations are provided in Supplemental Materials D.

Estimation of chloroprene metabolism in the human lung: Unfortunately, we found that the extremely low rates of chloroprene metabolism observed in the human lung (Himmelstein et al. 2004a) made parameter estimation for this tissue highly uncertain. The 95% confidence interval for human lung metabolism in the new MCMC analysis ranged from near zero (7.5E-23) to 0.44 L/hr/g microsomal protein, with a mean that was also near zero (1.5E-11). Therefore, we estimated the metabolism parameter for the human lung using the approach used in the USEPA (2011) risk assessment for methylene chloride, which relied on the PBPK model developed by Andersen et al. (1987). In that model, the K_m for metabolism in the human lung was assumed to be the same as the K_m in the human liver, and the V_{max} in the human lung was calculated from the V_{max} in the human liver using a parameter (A1) derived from the ratio of the specific activities for metabolism of 7-ethoxycoumarin, a well-studied CYP2E1 substrate, in liver and lung (Lorenz et al. 1984).

Model Simulations

The previously published version of the chloroprene PBPK model (Yang et al. 2012), which was written in the Advanced Continuous Simulation Language (ACSL), was translated into R, an open source programming language, to improve its portability. The R code for the model is included in Supplemental Materials E. The full model code, including the scripts for running the model, is available from the authors on request.

To model the experimental data from the nose-only inhalation exposures reported here, only the alveolar ventilation and cardiac output were altered. The average ventilation rate measured in the mice during the study was used to calculate an alveolar ventilation for use in the model,

assuming 2/3 of total ventilation is alveolar (Brown et al. 1997), and the cardiac output was then calculated by dividing the alveolar ventilation by the V/Q ratio from Marino et al. (2006), as described in the results.

Parameter sensitivity analysis was conducted with the model under two scenarios: (1) the prediction of blood concentrations in the mouse nose-only study, and (2) the prediction of dose metrics for the mouse bioassay exposures and for the human at 1 ppm continuous exposure. The results are displayed as normalized sensitivity coefficients (fractional change in prediction divided by fractional change in parameter) for parameters with a coefficient greater than 0.1 in absolute magnitude. A positive coefficient indicates the direction of change of the prediction is the same as the direction of change of the parameter. The parameters were changed by 1%, one at a time.

Dose metric calculations

Consistent with previous PBPK modeling of chloroprene (Himmelstein et al. 2004b, Yang et al. 2012), the dose metric calculated with the PBPK model is micromoles of chloroprene metabolized in the lung per gram lung per day. This dose metric was chosen because the lung is the tissue with the highest tumor incidence in the chloroprene inhalation bioassays (NTP 1998) and the carcinogenicity of chloroprene in rodents is believed to result from its metabolism to reactive epoxides in the target tissue (Himmelstein et al. 2004a, 2004b). The dose metric selected for chloroprene is consistent with the dose metrics used in previous PBPK-based risk assessments for both vinyl chloride (Clewett et al. 2001, USEPA 2000) and methylene chloride (Andersen et al. 1987, USEPA 2011), which were also based on the production of reactive metabolites.

The PBPK model was first used to simulate the NTP (1998) bioassay exposures (12.8, 32 and 80 ppm; 6 hours/day, 5 days/week) and calculate the corresponding target tissue dose metrics (in this case, average daily production of epoxide metabolites in the lung per gram lung). The PBPK model was then used to estimate the same target tissue dose metric in a human exposed continuously to chloroprene at a concentration of 1 µg/m³ for their lifetime.

3. RESULTS

Chloroprene Exposure Atmospheres

Chloroprene concentrations were monitored in the nose only chambers during the 13, 32, and 90 ppm exposures, as well as in the control nose-only tower. All three target concentrations were well within 10% of their nominal levels.

Plethysmography

Figure 2 shows the measured minute volumes for the three exposure groups and controls. The data is represented as average values (diamonds) with standard deviation error bars. The data is provided in Table S-5 in Supplemental Materials A. There is no evidence of a concentration-related effect of short-term exposure to chloroprene on ventilation in mice. The average ventilation rate across all four exposure groups, including controls, was 56.2 mL/min. The average body weight for the mice in the study was 22g; therefore, this ventilation rate equates to a model parameter for alveolar ventilation (QPC) of 39.4 L/hr/bw^{3/4}. The corresponding model value of QCC in this study is obtained by dividing QPC by the V/Q ratio of 1.45 for the mouse (Marino et al. 2006), yielding a value for QCC of 27.2 L/hr/bw^{3/4}, which compares well with the QCC of 24.2 estimated for mouse exposures to methylene chloride (Marino et al. 2006).

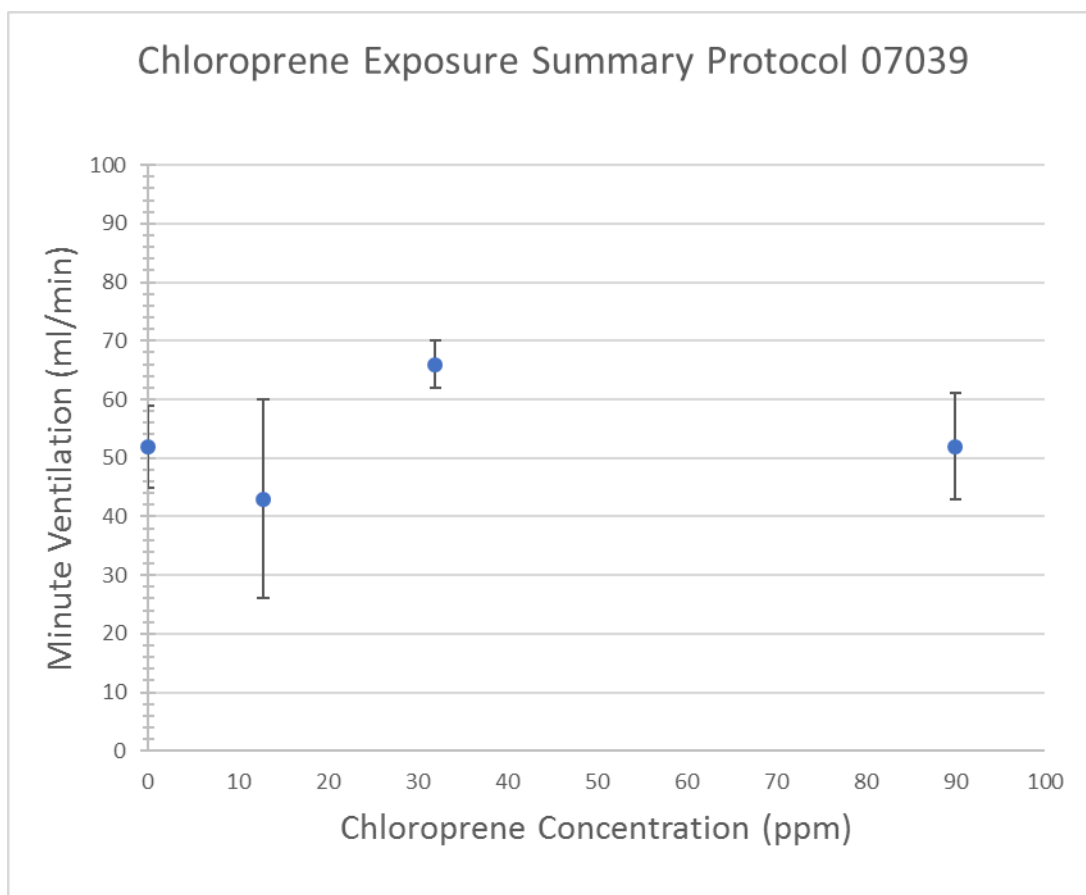


Figure 2. Measured minute ventilation during exposures.

Arterial Blood Chloroprene Concentrations

Figure 3 shows the average CD blood concentrations for all three single day exposures (Data are provided in Table S-6 of Supplemental Materials A). Average blood chloroprene concentrations are represented by the symbols with standard deviations for each treatment group represented with error bars.

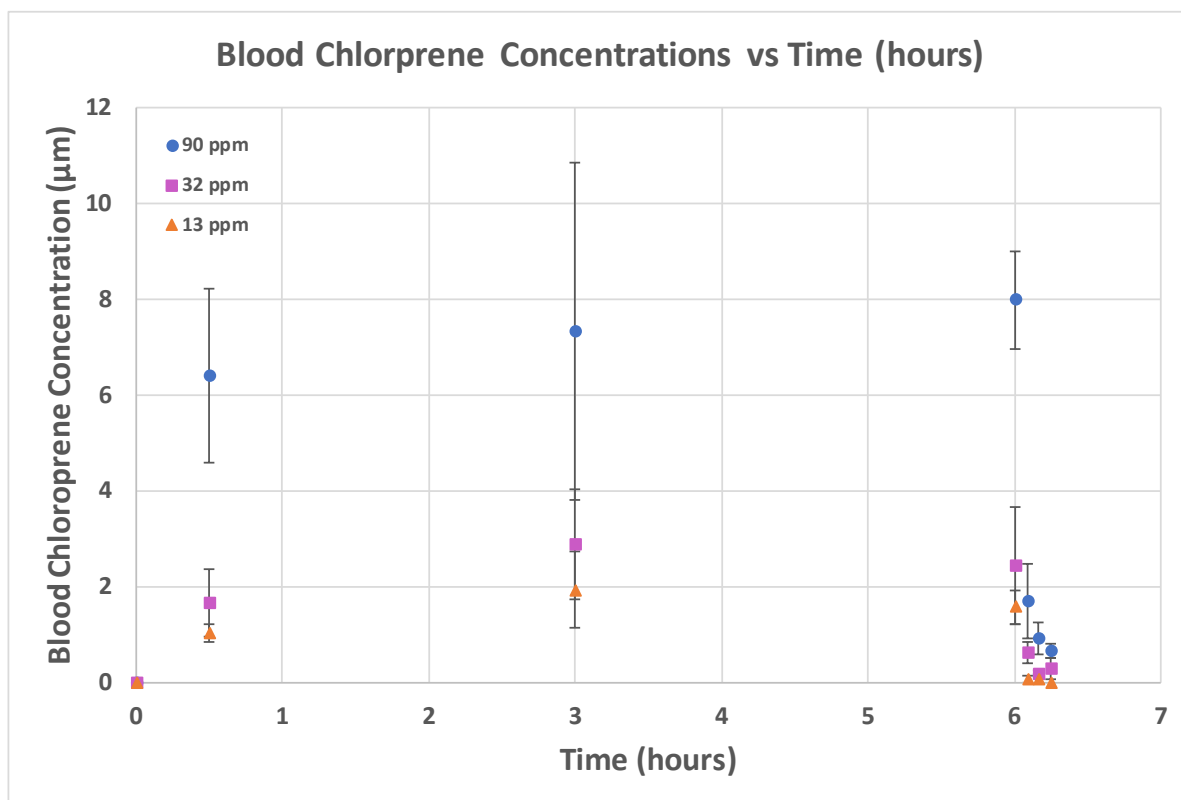


Figure 3. Arterial blood chloroprene concentrations during and following a single nose-only exposure of female B6C3F1 mice to chloroprene at 13, 32 and 90 ppm for 6 hours. Average blood chloroprene concentrations (symbols) and standard deviations (error bars) are shown for each treatment group.

PBPK Modeling of the Nose-Only Inhalation Study

The nose-only study described above was simulated with the chloroprene PBPK model using the parameters in Tables S1, S2, and S4, except for QPC and QCC, where the study-specific values derived from the plethysmography data were used. As shown in Figure 4, using only in vitro-derived metabolism and partitioning parameters the model predictions for arterial blood concentrations during and after the 6-hr chloroprene exposures are in good agreement with the data collected in the study, with model predictions generally lying within roughly a factor of two of the means of the experimental data. It was not necessary to adjust any of the model parameters to provide agreement with the new data.

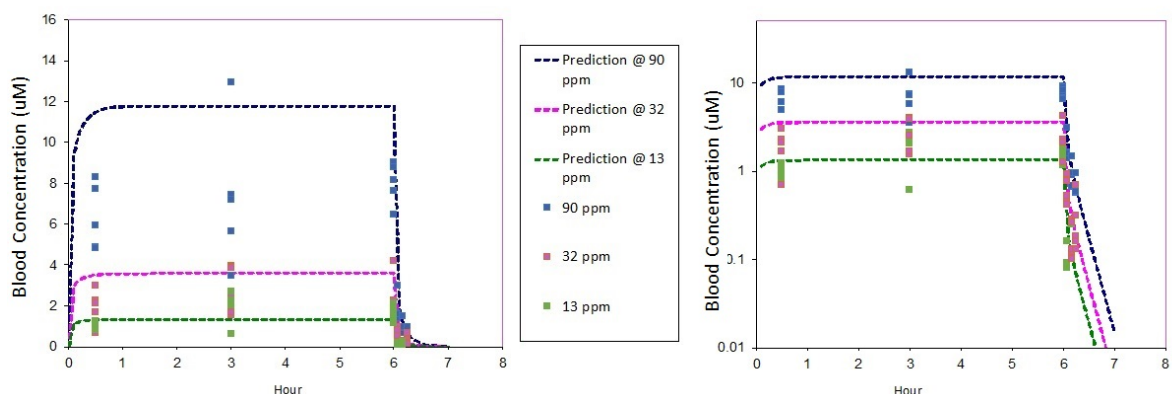


Figure 4. PBPK model predicted (dotted lines) and measured (symbols) arterial blood concentrations during and following 6-hr exposures of B6C3F1 mice to chloroprene at 13, 32 and 90 ppm. The same data and model predictions are shown using a linear y axis (left) and a logarithmic y axis (right). The linear plot provides a better comparison for concentrations, whereas the logarithmic plot provides a clearer comparison for the post-exposure clearance.

PBPK Model Parameter Sensitivity

As shown in Figure 5, when simulating the nose-only exposures only 4 model parameters have sensitivity coefficients greater than 0.1 in absolute magnitude: alveolar ventilation, cardiac output, blood:air partition coefficient and fractional blood flow to liver. All of these parameters were either directly measured or based on data from the literature, as described in the Methods, and can be considered to have low uncertainty. When predicting lung dose metrics in the female mouse (Figure 6), the sensitive parameters include the same parameters as those for the predictions of blood concentrations, with the addition of the parameters for lung metabolism and the body weight. The sensitive parameters for predictions of lung dose metrics in the human (Figure 7) are the same as those in the mouse, except that a single clearance parameter is used in the human due to the low rate of metabolism in the human lung. These analyses of the sensitivity of the model to uncertainty in its parameters suggest that performing a human in vivo validation study would be unlikely to provide a significant added value for model evaluation.

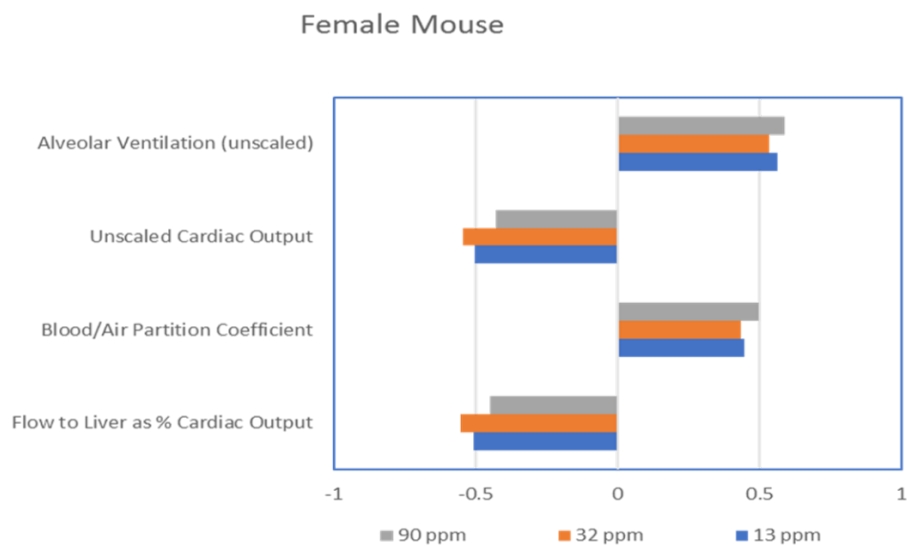


Figure 5. Parameter sensitivity coefficients for the chloroprene PBPK model for the prediction of arterial blood concentrations in the nose-only study.

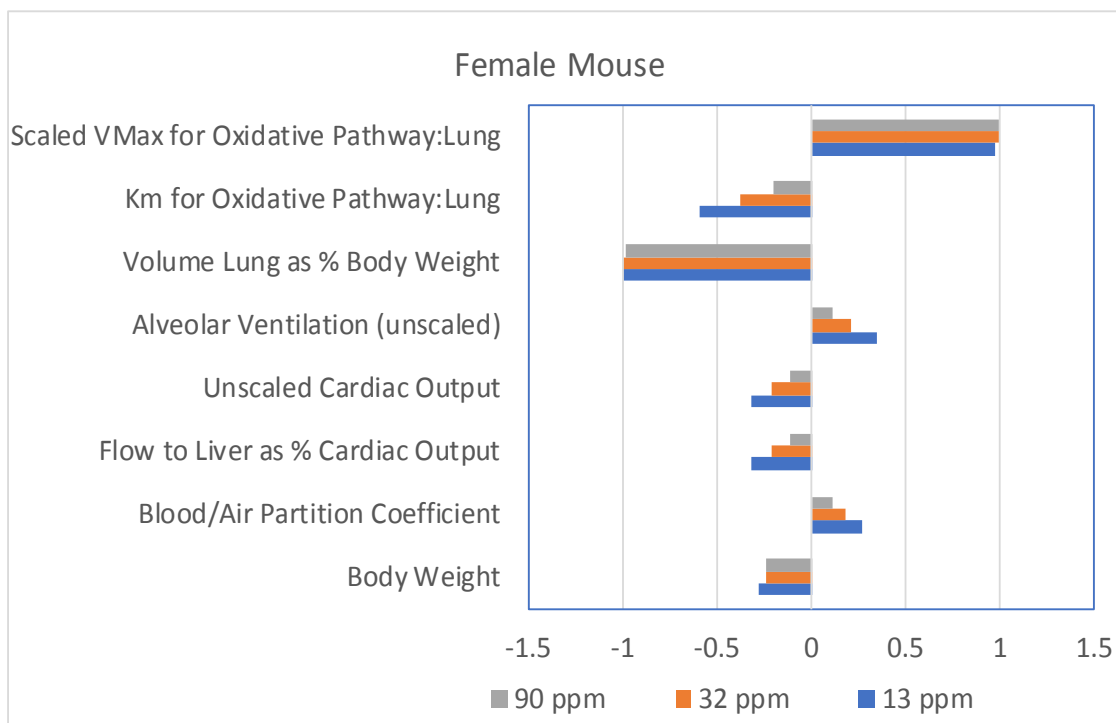


Figure 6. Parameter sensitivity coefficients for the chloroprene PBPK model for the prediction of lung dose metrics in the female mouse for exposures in the 2-year bioassay.

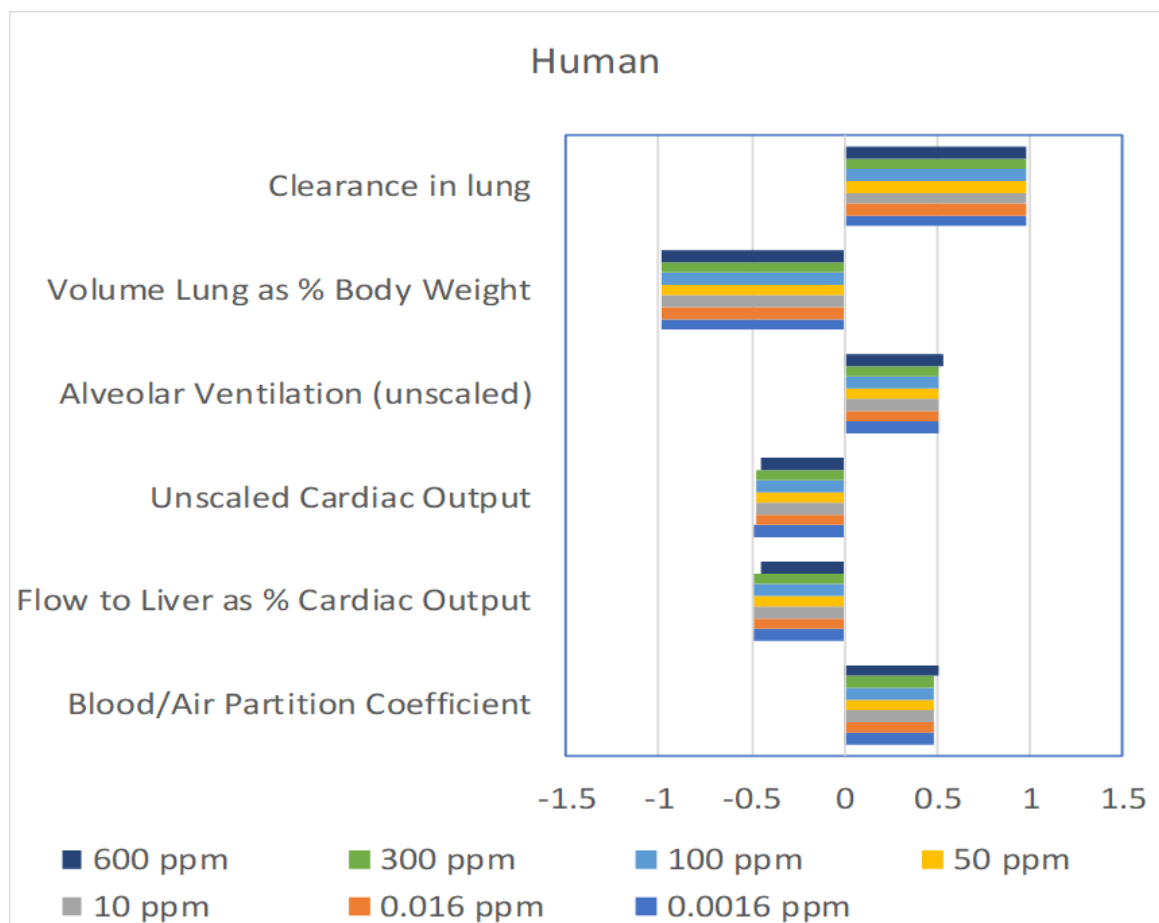


Figure 7. Parameter sensitivity coefficients for the chloroprene PBPK model for the prediction of lung dose metrics in the human for continuous exposure at 1 ppm.

PBPK-Based Dose Metrics for Chloroprene Lung Carcinogenicity

The dose metrics for lung metabolism in the female mouse bioassay and for human continuous exposure are shown in Table 1. These estimates were obtained with the chloroprene PBPK model using the parameters in Tables S1, S2, and S4.

Exposure	Concentration	Dose metric
Female mouse bioassay	12.3 ppm	1.20
	32 ppm	1.91
	80 ppm	2.52
Human continuous	100 ppm	3.95×10^{-2}
	10 ppm	3.14×10^{-2}
	1 ppm	1.02×10^{-2}
	0.1 ppm	1.32×10^{-3}
	0.01 ppm	1.36×10^{-4}
	1 ppb	1.36×10^{-5}
	1 $\mu\text{g}/\text{m}^3$	3.76×10^{-6}

Table 1. Dose metrics for lung metabolism (average mg metabolized per gram lung per day) in the female mouse bioassay and for human continuous exposures

As illustrated in Table 1, predicted dose metrics above 1 ppm increase less than linearly.

4. DISCUSSION

In this study we characterized the time course blood concentrations of chloroprene in female B6C3F1 mice during and following a single 6-hour nose-only inhalation exposure over the range of concentrations used in the NTP (1998) bioassays. These data, including both arterial whole blood concentrations and respiratory parameters (breathing frequency and tidal volume) during and after these exposures provide a reliable basis for evaluating the ability of the chloroprene PBPK model to predict in vivo pharmacokinetics in the bioassays. We have then applied the PBPK model to calculate dose metrics to support a risk assessment that considers species differences in pharmacokinetics. The use of a PBPK model for this purpose is consistent with the conclusion of the National Academy of Science (NRC 1987) that: “relevant PBPK data can be used to reduce uncertainty in extrapolation and risk assessment.”

It is important to note that, due to the low rates of metabolism in the in vitro assays for the rat and human lung, it is only possible to estimate a pseudo-first-order clearance for these tissues. Therefore, the original chloroprene model (Himmelstein et al. 2004b, Yang et al. 2012) used a linear description of metabolism in these tissues, which is only appropriate in the concentration range below the K_m in the lung, a parameter that is highly uncertain in the rat and human. Thus model-based metabolism predictions for human exposures significantly greater than 1 ppm would greatly overestimate the associated risk. One approach for dealing with the inability to estimate the value of K_m in the human lung is to use the value of K_m estimated in the human liver. This approach was used in the PBPK model for methylene chloride (Andersen et al. 1987) and in the present analysis. The impact of saturable metabolism on human dose metric predictions is shown in Figure 8. Without estimating a value for K_m , the model-predicted risks above 1 ppm would continue to increase at a biologically implausible rate.

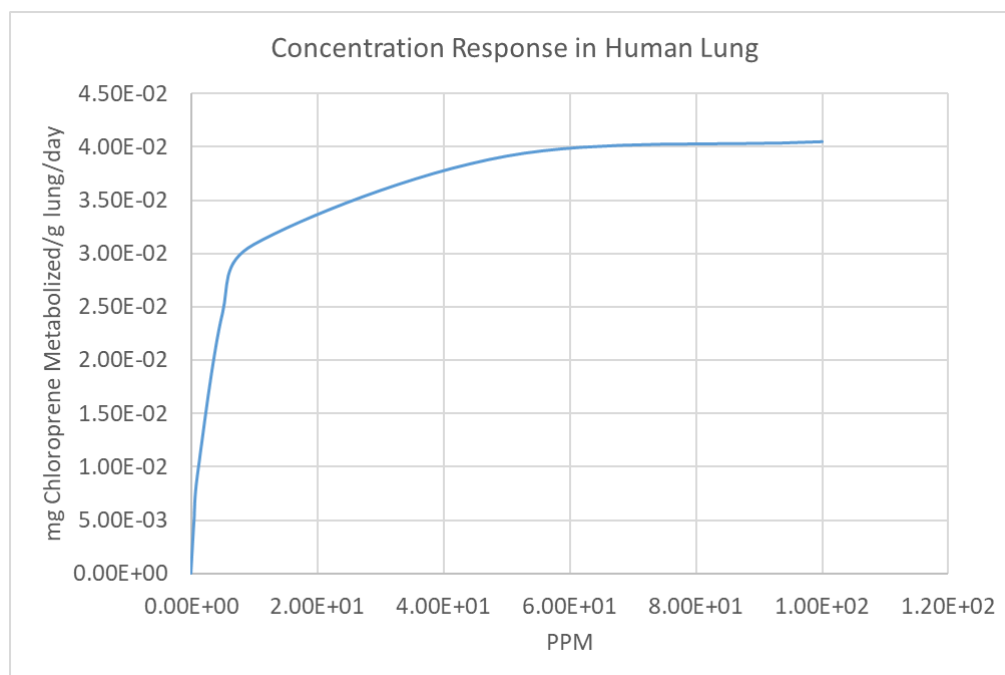


Figure 8. Inhaled concentration dependence of lung metabolism in the human for continuous exposures to chloroprene predicted with the PBPK model.

Interestingly, comparison of the K_m s for chloroprene in liver and lung for male and female mice (Table S-3), which are based on the strongest data sets for estimating K_m s, suggests that K_m may be higher (lower affinity) in the mouse lung than in the mouse liver. This difference in apparent affinities in mouse liver and lung is consistent with differences in the relative tissue abundances of the murine CYP2E1 and CYP2F isozymes, both of which exhibit high affinities for chlorinated alkenes (Yoon et al. 2007). Whereas CYP2E1 is the predominant high affinity isozyme in the mouse liver, CYP2F is the predominant high affinity isozyme in the mouse lung (Yoon et al. 2007) and, consistent with the estimated K_m s for chloroprene, the affinity of rCYP2E1 is roughly 3-fold higher (lower K_m) than rCYP2F2 (Simmonds et al. 2004). However, since there is no evidence of CYP2F activity in the primate lung (Baldwin et al. 2004), no difference in K_m s in the human lung and liver would be expected, so the estimation of human lung K_m based on the human liver K_m is appropriate.

Not unexpectedly, in our re-analysis we found that the extremely low rates of chloroprene metabolism observed *in vitro* in the human lung (Himmelstein et al. 2004a) made parameter estimation for this tissue highly uncertain. The 95% confidence interval for human lung metabolism in the new MCMC analysis ranges from near zero ($7.5E-23$) to 0.44 L/hr/g microsomal protein, with a mean that is also near zero ($1.5E-11$). Therefore, we estimated the metabolism parameter for the human lung using the same approach as the USEPA (2011) risk assessment for methylene chloride; that is, the K_m for metabolism in the human lung was assumed to be the same as the K_m in the human liver, and the V_{max} in the human lung was calculated from the V_{max} in the human liver using a parameter (A1) derived from the ratio of the specific activities for metabolism of 7-ethoxycoumarin, a well-studied CYP2E1 substrate, in liver and lung (Lorenz et al. 1984). Using the human value of A1 (0.00143), together with the estimated values of V_{max} and K_m in the human liver from the MCMC analysis (0.053 $\mu\text{mol/hr/mg}$ protein and 0.38 $\mu\text{mol/L}$), results in a metabolic clearance in the lung of 0.20 L/hr/g microsomal protein. This human lung metabolism estimate is similar to the value of 0.32 L/hr/g microsomal protein previously estimated for chloroprene by Yang et al. (2012) and is within the confidence interval estimated by our new analysis of the *in vitro* data. In support of the applicability of A1 to chloroprene, the value of A1 in the male mouse (0.414) from Lorenz et al. (1984) is close to the ratio of the *in vitro* V_{max} in the lung and liver of the male mouse in our new analysis (0.56, see Table S-3).

Selection of Dose Metric

The dose metric calculated with the PBPK model in this analysis is micromoles of chloroprene metabolized in the lung per gram lung per day (Himmelstein et al. 2004b, Yang et al. 2012). This dose metric was chosen because (1) the lung is the tissue with the highest tumor incidence in the chloroprene inhalation bioassays (NTP 1998) and (2) the carcinogenicity of chloroprene in rodents is believed to result from its metabolism to reactive epoxides in the target tissue (Himmelstein et al. 2004a, 2004b). The dose metric selected for chloroprene is consistent with the dose metrics used in previous PBPK-based risk assessments for both vinyl chloride (Clewett et al. 2001, USEPA 2000) and methylene chloride (Andersen et al. 1987, USEPA 2011), which were also based on the rate of production of reactive metabolites. The dose metric selected for the liver carcinogenicity of vinyl chloride was total mg vinyl chloride metabolized per kg liver per day, representing the production of the reactive chloroethylene epoxide. Due to the presence of chlorine in the epoxides generated from the metabolism of chloroprene, they are considered likely to have a reactivity comparable vinyl chloride (Haley 1978, Plugge and Jaeger 1979). The methylene chloride dose metric was average daily metabolism by the glutathione conjugation

pathway in the lung per gram lung, which was selected based on evidence that the carcinogenicity of methylene chloride was associated with the local production of a reactive metabolite from the glutathione conjugate of methylene chloride. As with vinyl chloride and chloroprene, the assumption inherent in the dose metric was that the reactive metabolite would be completely consumed within the tissue where it was generated (Andersen et al. 1987).

Himmelstein et al. (2004b) have previously demonstrated that using the PBPK dose metric is able to harmonize the dose-responses for lung tumors in mice, rats and hamsters. However, they only had metabolism data for male animals. Figure 9 shows an update of the analysis from Himmelstein et al. (2004b) that includes the results for the female mouse and rat. While the revised PBPK model is still able to demonstrate the consistency of the tumor incidence across male animals of different species and strains, female mice exhibit a higher tumor incidence than male mice at the same rate of lung metabolism.

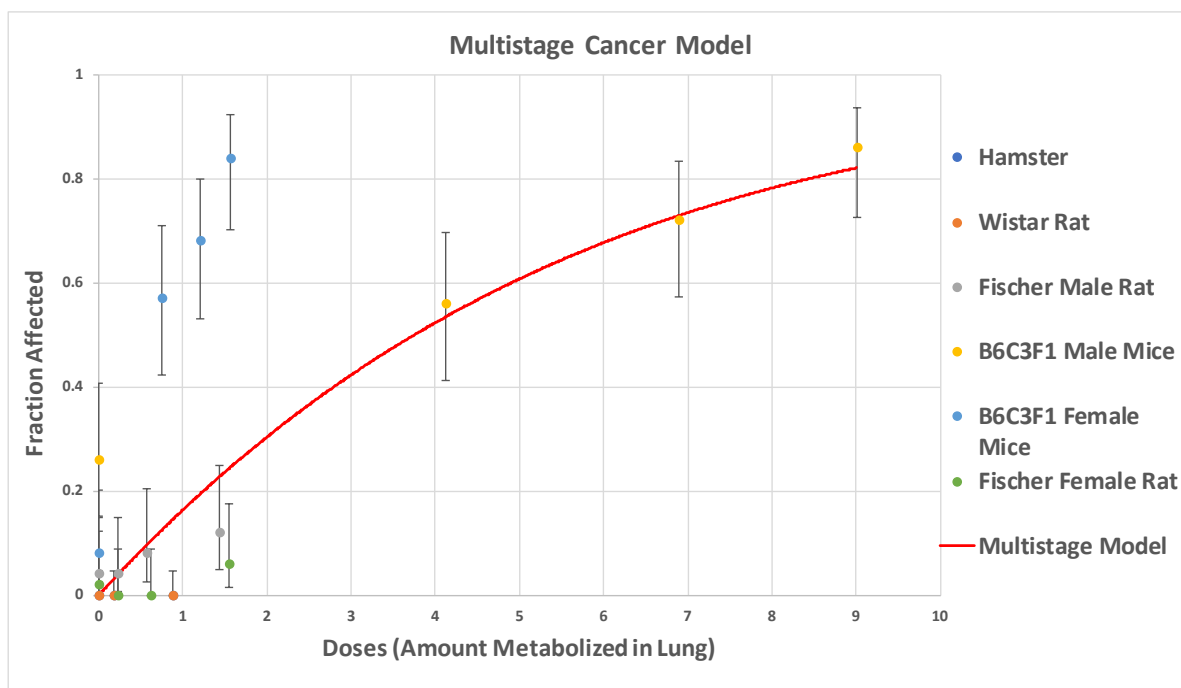


Figure 9. Comparison of Dose-Response for Lung Tumors in Chloroprene Bioassays in Rodents.

This discrepancy could indicate either of two possibilities: (1) the selected dose metric, rate of metabolism of chloroprene in the lung, is incorrect, or (2) the female mouse lung is more sensitive to the effects of chloroprene metabolites than the male mouse lung. Relatively few studies have been conducted to explore gender differences in the responses to chemical insult in the mouse lung. However, Yamada et al. 2017, provides evidence of a proliferative response of Club cells to the toxicity of permethrin in the female mouse lung that is not observed in the male mouse lung, and studies of naphthalene lung toxicity have demonstrated a greater sensitivity of the female mouse lung to both acute and repeated toxicity (Van Winkle et al. 2002, Sutherland et al. 2012). The greater susceptibility to a proliferative response to lung toxicity in the female mouse appears to result from gender differences in the tissue response to damage rather than metabolism (Laura Van Winkle, personal communication). A study of the genomic responses in the lungs of female mice and rats to inhaled chloroprene (Thomas et al. 2013) also demonstrated

a greater pharmacodynamic sensitivity of the female mouse. In this study, female mice and rats were exposed for up to three weeks to inhaled chloroprene concentrations that were chosen to result in similar rates of epoxide production in the two species. The study found that while the most sensitive tissue responses occurred at similar values of the metabolism dose metric, transcriptional evidence of oxidative stress occurred at much lower concentrations in the female mouse. The more sensitive response of the female mouse to oxidative stress and to a proliferative response may underlie the apparent potency difference indicated by Figure 9. Using the metabolism dose metric appropriately considers the greater sensitivity of the female mouse in a manner that is health protective, since the greater sensitivity of the female mouse results in a lower BMDL01 than would be obtained from the male mouse.

As described in Supplemental Materials F, a steady-state analysis demonstrates that the chloroprene PBPK dose metric based on total metabolism per gram lung is conservative (i.e. a dose metric that also considered clearance would result in similar or lower risk estimates). The toxicity and associated carcinogenicity from chloroprene is expected to be related to the average concentration of the epoxides in the target tissue over the duration of exposure; therefore, the differences across species will be determined by the relationship between production and clearance. Due to the expectation that the rearrangement of CEO to reactive aldehydes is spontaneous and not enzymatically catalyzed, and that the reaction of epoxides with glutathione is primarily related to direct GSH conjugation rather than enzymatic conjugation via glutathione transferases, clearance by these pathways would be expected to be identical across species, as was the case for methylene chloride, where the clearance of the chloromethylglutathione metabolite was non-enzymatic and rapid. The only other clearance pathway, enzymatic hydrolysis of chloroprene by epoxide hydrolase, has been shown to be slower in mouse lung compared to human lung (Himmelstein et al. 2004a). Thus, the total clearance is expected to be similar or greater in the human compared to the mouse, and the use of the dose metric based solely on production would provide a conservative (similar or higher) estimate of human risk compared to a dose metric that also considered clearance.

The risk assessment for vinyl chloride (USEPA 2000) demonstrated that the use of a PBPK model to estimate target tissue dose (based on total metabolism per gram liver per day) was able to produce similar human risk estimates using data from animal bioassays and human occupational exposures. As a similar test of the chloroprene PBPK model to support cross-species extrapolation, Allen et al. (2014) used a statistical maximum likelihood approach to compare risk estimates obtained using external (air concentration) and internal (PBPK model estimated) metrics for the female mouse bioassay and human occupational exposures. The analysis concluded that if inhaled concentration was used as the dose metric, the estimates of human cancer risk using animal and human data were statistically significantly different, whereas using the PBPK metric consistent risk estimates were obtained across species. As with vinyl chloride, the use of the PBPK-based metric effectively reconciled the differences in mouse and human low-dose risk estimates.

Use of In Vitro Metabolism Data

The most notable aspect of the chloroprene PBPK model is that, apart from the physiological parameters, the parameters in the model are based on data derived solely from in vitro studies. The PBPK model for chloroprene is structurally similar to the PBPK model for methylene chloride (Andersen et al. 1987) and, just as in the case of the methylene chloride risk assessment, model predictions needed to support a risk assessment are critically dependent on parameters that can only be derived from in vitro metabolism experiments.

In their analysis of in vitro data on chloroprene metabolism, Yang et al. (2012) employed both a standard frequentist approach (referred to in their analysis as a “deterministic” approach) and an approach that used a Markov Chain Monte Carlo (MCMC) method (referred to as a “probabilistic” approach) with non-informative prior distributions for all estimated parameters. The use of non-informative priors allows this Bayesian approach to be interpreted from a frequentist perspective. As stated in the Yang et al. (2012) document, the two methods were compared to demonstrate that they provided consistent estimates of metabolic parameter values. Yang et al. (2012) then relied on the MCMC-based estimates for developing dose metrics for chloroprene exposures in mouse, rat and human. Because it seeks a global optimum using a probabilistic direct search algorithm, MCMC is less likely than deterministic search algorithms to converge on a local optimum. Moreover, when used with non-informative priors, as in Yang et al. (2012), the posterior distribution represents the likelihood distribution for the parameter, and the mode of the distribution represents the maximum likelihood estimate (MLE). As pointed out in Chiu et al. (2007), the Bayesian approach, in principle, yields a more global characterization of parameter uncertainty than the local, linearized variance estimates provided by traditional optimization routines, which should be viewed as lower bound estimates of true parameter uncertainty. Because of its superior properties, we have also relied on the MCMC approach in our re-analysis of the original in vitro metabolism data.

The key difference between the MCMC analysis performed in this study and the original analysis (Yang et al. 2012) was that this re-analysis included an additional parameter (Kgl) for the in vitro experiments, representing the potential for a mass transport limitation for uptake of chloroprene from the air in the metabolism vials. To evaluate the impact of our re-analysis of the in vitro metabolism data using Kgl on predicted risk estimates, the PBPK model was also used to calculate dose metrics using the previously published metabolism parameters from Yang et al. (2012). Again, due to the high uncertainty the human lung metabolism parameter, the approach using A1 from Andersen et al. (1987) was applied. The results with the two parameterizations are compared in Table 2.

Exposure	Concentration	Dose Metrics Yang et al. 2012 parameters	Dose Metrics Re-estimated parameters
Female mouse bioassay	12.3 ppm	0.75	1.20
	32 ppm	1.20	1.91
	80 ppm	1.57	2.52
Human continuous	1 µg/m ³	2.7x10 ⁻⁶	3.76x10 ⁻⁶

Use of In Vitro Metabolism Data

PBPK modeling has now been applied in risk assessments for a variety of environmental chemicals by regulatory agencies worldwide. The development of these models has typically required the use of in vivo experimental animal and/or human data to estimate key kinetic parameters such as uptake, metabolism and elimination. Some agencies also require the use of separate in vivo data to demonstrate model validity. However, it has become increasingly difficult to conduct controlled exposures of human subjects to chemicals of concern, other than for pharmaceuticals. The need for live animal studies is also being challenged, particularly in the

EU, due to both ethical and practical (cost, throughput) concerns. Therefore, requirements for in vivo testing will increasingly limit the potential application of PBPK modeling in risk assessment, and agencies will need to consider whether in vivo validation data is truly necessary for assessing the fitness of a model for the specific purpose of its use in a particular risk assessment.

The original chloroprene PBPK model (Himmelstein et al. 2004b) was not used by USEPA (2010) because the agency considered it necessary to have blood or tissue time course concentration data from an in vivo study to adequately validate the model. The study reported here was conducted to address this requirement and we have now demonstrated that the chloroprene PBPK model accurately simulates these in vivo blood time course validation data.

No in vivo validation data for chloroprene is available in the human, and it is unlikely that such a study could be performed given the current classification of chloroprene as “likely to be a carcinogen” (USEPA 2010). However, the sensitivity analyses reported here suggest that such a study would not provide significant added value for demonstrating that the PBPK model is fit for purpose for a chloroprene risk assessment. The validity of the model instead derives from the biological validity of the physiological and biochemical underpinnings of the model structure and parameters. The key parameters for performing a risk assessment for chloroprene are those for lung metabolism, and a human in vivo study would not be able to provide informative data for those parameters. As shown in Figure 5, blood concentrations of chloroprene associated with inhalation are insensitive to lung metabolism, and depend only on alveolar ventilation, cardiac output, blood:air partition coefficient and fractional blood flow to liver.

The limited value of human in vivo data for determining whether a PBPK model is fit for purpose in a risk assessment based on target tissue metabolism was also an issue during the development of the PBPK model of methylene chloride (Andersen et al. 1987), where a similar dose metric was used: average daily metabolism of methylene chloride by glutathione transferase (GST) in the lung per gram lung. Although the model accurately reproduced blood and exhaled air concentration time-course data from multiple studies with human subjects, the in vivo data were not adequate to estimate the rates of GST metabolism in the liver and lung. Instead, it was necessary to estimate the rate of GST metabolism in the human liver by allometric scaling from animal data (Andersen et al. 1987), and to then estimate the rate of GST metabolism in the human lung using the ratio of specific activities for GST metabolism in liver and lung measured in vitro by Lorenz et al. (1984). This same approach was used in the chloroprene modeling documented in this report.

At the time the methylene chloride PBPK model was developed, the use of in vitro data to predict in vivo metabolism was a relatively new concept, but in the intervening years it has become common practice both for pharmaceuticals (Rostami-Hodjegan 2012) and environmental chemicals (Yoon et al. 2012). Nevertheless, regulatory agency acceptance of PBPK models that are not based primarily on in vivo data still presents a challenge. Going forward it will be important to develop a consensus on standard practices for IVIVE of metabolism in PBPK modeling to assist agencies in their evaluations. Additionally, PBPK model evaluations should make greater use of uncertainty analyses to estimate the potential reduction in model uncertainty associated with the collection of additional data; that is, to determine the added value of a proposed study.

5. CONCLUSION

A PBPK model of chloroprene that relies solely on data from in vitro studies for its metabolism parameters accurately predicts the in vivo time course for chloroprene in the blood of female mice exposed by nose-only inhalation at the 3 concentrations used in the chloroprene 2-year cancer bioassay. This PBPK model has been used to estimate dose metrics for the metabolism of chloroprene to reactive epoxides in the lung target tissue of mice and humans to support an inhalation cancer risk assessment for chloroprene. Large differences between PBPK-based risk estimates and estimates based on inhaled concentration have been seen in previous inhalation risk assessments for chemicals where toxicity results from the production of reactive metabolites (Andersen et al. 1987, Clewell et al. 2001). The present PBPK model follows the same approach used in these previous PBPK models and incorporates the best available science to describe the impact of species differences in metabolism on the potential cancer risk associated with chloroprene inhalation.

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Supplemental Materials

- A. Supplemental Tables
- B. Re-estimation of Metabolism Parameters
- C. IVIVE Literature Review
- D. Metabolism Parameter Calculations
- E. PBPK Model Equations
- F. Considerations in choosing a dose metric